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Hull Cupules of Chiton Eggs: Parachute Structures and Sperm Focusing Devices?

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Abstract. The extracellular hull of chiton eggs is often elaborated into cupules or spines that may be open or closed to the external environment. Scanning electron microscopy was used to examine the location of fertilizing sperm in eggs that had been exposed to a dilute sperm suspension to create natural fertilization or to a sperm concentrate to induce polyspermic egg penetration. The effect of cupules on sinking rates was tested in cupulous (free-spawning) and non-cupulous (brooding) species, by timing descent of eggs over a fixed distance in a large container of seawater. Densities of eggs were compared on Percoll gradients and found to be similar. It was found that hull cupules focus the sperm to specific regions of the egg surface in both brooding and free-spawning species. Furthermore, protruding cupules act as parachute structures that can significantly reduce sinking rates.

Introduction

In a majority of chitons, the extracellular egg hull is elaborated into complex cupules or spines that project outwards from the surface of the egg (see review by Pearse, 1979). The mechanism of sperm entry was misunderstood for many years, partly because hull elaborations made it difficult to visualize sperm-egg interactions, and partly because accumulated ultrastructural evidence favored the lack of an acrosome, and there was no obvious means of sperm entry (Pearse and Woollacott, 1979; Russell-Pinto *et al.*, 1983, 1984; Sakker, 1984; Al-Hajj, 1987; Hodgson *et al.*, 1988). The discovery of a tiny acrosome at the tip of the nuclear filament in *Tonicella lineata* (Buckland-Nicks *et al.*, 1988a), the documentation of fertilization in this species (Buckland-Nicks *et al.*, 1988b), and the subsequent demonstration that similar acrosomes are present in five different sub-families of chitons (Buckland-Nicks *et al.*, 1990), indicated that with the exception of one primitive sub-family (Hodgson *et al.*, 1988), there is a common mechanism of fertilization among most chitons. However, there are striking differences in the structure of the egg hull of chitons. For example, hull cupules may be open or closed to the external environment; they may be blunt or spinous, reduced to plates or bumps, or be totally absent (see reviews by Pearse, 1979; Eernisse and Reynolds, 1993).

In *T. lineata*, the cupules are opened by follicle cell retraction when the eggs ripen and sperm are attracted inside the cupules to fertilize each egg (Buckland-Nicks *et al.*, 1988b); but where do the sperm enter in closed cupule species? In brooding forms such as *Lepidochitona fernaldi* and *L. thomasi*, the cupules are of the closed type; furthermore, they are reduced in these species to flattened plates (Eernisse, 1988). This variation in cupule size, shape, and structure may have profound influences on the site and mechanism of fertilization, the sinking rates, adhesion, and cohesion of eggs, as well as the numbers of eggs that can be brooded by brooding species. This study examines the role of hull cupules in focusing sperm to a particular region of the egg surface, as well as their influence on sinking rates in free-spawning *versus* brooding species.

Materials and Methods

Specimens of the free-spawning species *Mopalia lignosa* (Gould, 1846), *Mopalia ciliata* (Sowerby, 1840), *Mopalia muscosa* (Gould, 1846), and *Lepidochitona dentiens* (Gould, 1846) were obtained in May 1989 and 1990 from tidepools and beneath rocks in the intertidal zone at

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Eagle's Cove, San Juan Island, Washington. Specimens of the brooding species *L. fernaldi* (Eernisse, 1986) were obtained in May 1990 from tidepools in association with the barnacle *Semibalanus cariosus* (Pallas, 1788) and the sea anemone, *Anthopleura elegantissima* (Brandt, 1835), at Deadman Bay on the west coast of San Juan Island. Specimens of *Chaetopleura apiculata* (Say, 1834) were purchased from Gulf Specimen Co., Panacea, Florida, and shipped to San Juan Island in early June 1990. Animals were maintained at Friday Harbor Labs in separate dishes on a running seawater table at about 10°C until spawning occurred naturally. No attempt was made to induce spawning artificially. Some individuals of a species were kept in the same tank to encourage natural fertilization. When polyspermy was required, unfertilized eggs of *M. lignosa, M. muscosa, M. ciliata, C. apiculata* and *L. dentiens* were collected and fertilized with sperm dissected from males of the same species. Eggs of *L. fernaldi* were dissected from the ovary or removed from the pallial groove with a toothpick following spawning. Eggs were washed in two changes of 0.45 μm millipore-filtered seawater prior to sedimentation experiments, to remove mucus.

Scanning electron microscopy

For scanning electron microscopy fertilized eggs were fixed in cold 2.5% glutaraldehyde in 0.45 μm millipore-filtered seawater at pH 8.0 (adjusted by adding 1 *N* NaOH to 25% glutaraldehyde prior to mixing 1:9 with seawater) for 3 h followed by a rinse in 2.5% sodium bicarbonate buffer at pH 7.2 and post-fixation with cold 1% osmium tetroxide in 1.25% sodium bicarbonate buffer at pH 7.2 (final concentrations). Eggs were washed in distilled water and dehydrated in a graded ethanol series to 100% ethanol. The ethanol was gradually replaced by amyl acetate to 100%, with three changes in pure amyl acetate, prior to critical point drying in teflon "microporous specimen capsules" (S.P.I. supplies). Eggs were tapped out onto aluminum stubs coated with double-sided sticky tabs. Some eggs were rolled on the sticky tab to remove cupules and reveal internal structure and sperm-egg interactions. Following these pre-treatments, the eggs were sputter-coated with gold and examined in a Cambridge S250 or S150 scanning electron microscope.

Sedimentation velocity

Sinking rates of eggs of *M. ciliata* and *L. fernaldi* were tested by dropping individual eggs into the center of a 5-l beaker of 1 μm filtered seawater and timing their descent over a distance of 25 cm between two marks on the side of the beaker. The upper mark was approximately two inches below the surface of the water, which allowed eggs to reach terminal velocity before timing began. Wall effects were assumed to be minimal and in any case would cancel out in comparisons between species.

Percoll gradients

Densities of eggs of *M. ciliata* and *L. fernaldi* were compared empirically by centrifugation on paired Percoll (Pharmacia, Sweden) gradients. Ten milliliter aliquots of different concentrations of Percoll ranging from 10 to 70% Percoll were made up in 0.45 μm millipore-filtered seawater. Beginning with 70% Percoll, each aliquot was drawn up into a 10 ml disposable plastic pipette using a pi-pump (Fisher), and 5 ml of it was gently layered into each of two slanting, 50-ml capacity, pyrex centrifuge tubes. The final result was a Percoll gradient ranging from 70% at the bottom of the tube to 10% at the top. Samples of eggs in 5 ml of 0.45 μm millipore-filtered seawater were pipetted on top of each gradient, and the gradients were transferred to a bench top centrifuge (being very careful to avoid bumping the tubes) and spun at 400 G's for 10 min. Five replicates were done for each species (eggs were recovered and re-used for *L. fernaldi*) with equivalent results each time. In one instance the centrifuge was stopped and the tubes examined after 2 min. The resulting distribution of eggs was photographed against a white background with Kodak 2415 Technical Pan film.

Results

Evidence for the influence of hull cupules on the site of fertilization

In ripe eggs of *Mopalia* spp. the hull cupules are open to the external environment (Figs. 1, 3). Sperm rapidly located the open cupules and swam inside one of seven channels, at the base of which they penetrated the hull (Figs. 2, 4). The fact that this is the main site of fertilization in this species was demonstrated by exposing eggs to high sperm concentrations which induced polyspermic egg penetration. When these eggs were rolled on sticky tape to remove cupules, numerous sperm were found penetrating the hull inside the cupules, but very few were seen penetrating in the intercupule area (Fig. 4).

Figures 1–4: Scanning electron micrographs of fertilized eggs of *Mopalia* spp.

Figure 1. Fertilized egg of *M. ciliata*, demonstrating open cupules. Scale bar = 50 μm.

Figure 2. Naturally fertilized egg of *M. lignosa* rolled on sticky tape to remove some cupules and demonstrate subdivision of each cupule into seven channels. Sperm (arrow) can be seen penetrating the hull within some channels. Scale bar = 20 μm.

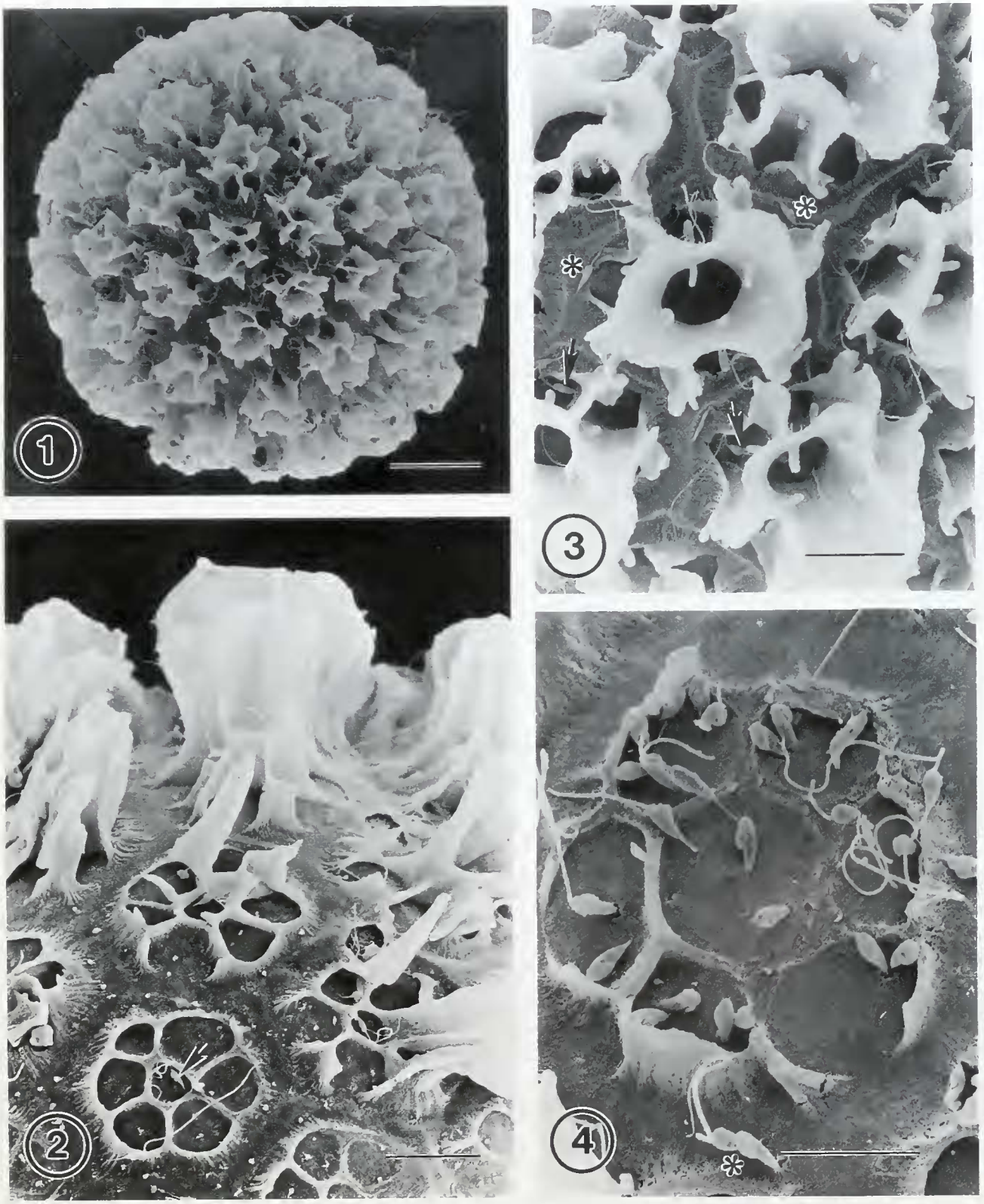


Figure 3. Hull cupules of naturally fertilized egg of *M. muscosa*. Note sperm inside cupule (arrows), and few sperm between cupules that are not penetrating the hull (asterisks). Scale bar = 15 μm.

Figure 4. Egg of *M. lignosa* artificially fertilized with sperm concentrate to create polyspermy. Few sperm have penetrated hull between cupules (asterisk) but numerous sperm can be seen penetrating within cupules. Scale bar = 10 μm.

The ripe eggs of *Lepidochitona dentiens* are permanently closed (Fig. 5), blocking sperm entry. Instead, sperm were focused to the intercupule area which represents roughly 20% of the egg surface. The removal of cupules in this species revealed that sperm did not gain access to the inside of cupules (Fig. 6). Instead, sperm were found in abundance in the intercupule area (Fig. 7). On closer examination, the intercupule area was found to be characterized by a series of micropores. Sperm appeared to penetrate the hull via individual micropores (Fig. 8). Micropores were not found in the intercupule area of *M. muscosa* (Fig. 3) or *M. lignosa* eggs (Fig. 4), but they were found in this region in eggs of the brooder *L. fernaldi* (Fig. 9), which, like *L. dentiens*, has closed hull cupules. Furthermore, occasional elongate microvilli were seen projecting from the micropores (Fig. 10).

Preliminary examination of spinous-hulled eggs of *Chaetopleura apiculata* (Fig. 11), showed that after the spines had unwound during maturation of the egg (Fig. 12), they remained closed to the external environment, yet hollow inside (Fig. 13). This condition resembles that of the closed-cupule species, *L. dentiens*. Sperm did not gain access to the inside of the spines. Unfortunately, most eggs were damaged and it was not possible to view the intercupule area to assess sperm binding or penetration in this region, nor to discover the presence or absence of micropores.

Evidence for the influence of hull cupules on sinking rates

Sinking rates of eggs of *M. ciliata* and *L. fernaldi* were measured by dropping individually 20 eggs of each species into the center of a 5-l beaker of seawater and timing their sinking rates between two marks, 25 cm apart. The mean sinking rate for *M. ciliata* was 330 μm/s (S.E. = 0.48); and for *L. fernaldi* was 1930 μm/s (S.E. = 0.10) $\{P < 0.0001\}$. Eggs of the open cupule species sank almost six times more slowly than eggs of the brooding species.

To test whether this was due largely to differences in overall density, the eggs of *L. fernaldi* and *M. ciliata* were centrifuged on Percoll gradients for 10 min at 400 G's. However, the results presented in Figure 14 show that egg density was roughly the same. What was interesting was that if the centrifuge was stopped after 2 min, eggs of *L. fernaldi* had already reached their final level in the gradient, whereas those of *M. ciliata* had only migrated a fraction of this distance (Fig. 14). After 10 min, eggs of both species had reached the same level in the gradient, which did not change with longer centrifugation times.

The diameter of the egg core (248 μm), excluding the hull, of an *L. fernaldi* egg, was slightly larger than that of *M. ciliata* (231 μm). However, the total diameter, taking into account the hull and hull cupules, was 25% less in *L. fernaldi* eggs (292 μm) than those of *M. ciliata* (363 μm). The actual volume of eggs was calculated to be approximately 9.27×10^{-3} mm^3 for *L. fernaldi* and 8.04×10^{-3} mm^3 for *M. ciliata* (volume of cupules was calculated by estimating the volume of a single cupule compressed into a rectangle of known dimensions and multiplying by the number of cupules on the egg). Thus the cupulous egg of *M. ciliata* has a smaller actual volume, which is distributed over a larger effective volume.

Discussion

Do hull elaborations focus the sperm?

In the majority of chitons, the egg hull is elaborated into a series of cupules or spines which cover much of the egg surface. This covering of cupules restricts sperm access to some parts of the egg in some species, while focusing sperm to particular regions in other species. Cupules may be open or closed at maturity. In open cupule species, the inside of the cupule is exposed when the follicle cell covering it has retracted (Buckland-Nicks *et al.*, 1988b, and this study). The sperm are attracted inside the cupules and penetrate the egg at the base of these cupules. Sperm are not attracted to individual cupules when still covered by follicle cells, and infrequently penetrate the hull in the intercupule area (Buckland-Nicks *et al.*, 1988b; and this study). Miller (1977) has shown that a sperm chemoattractant is released by some chiton eggs, including *Mopalia* spp. My observations suggest that this chemoattractant is probably released from within hull cupules, following follicle cell retraction. The result is that sperm are focused to a restricted area of the egg surface inside the cupules.

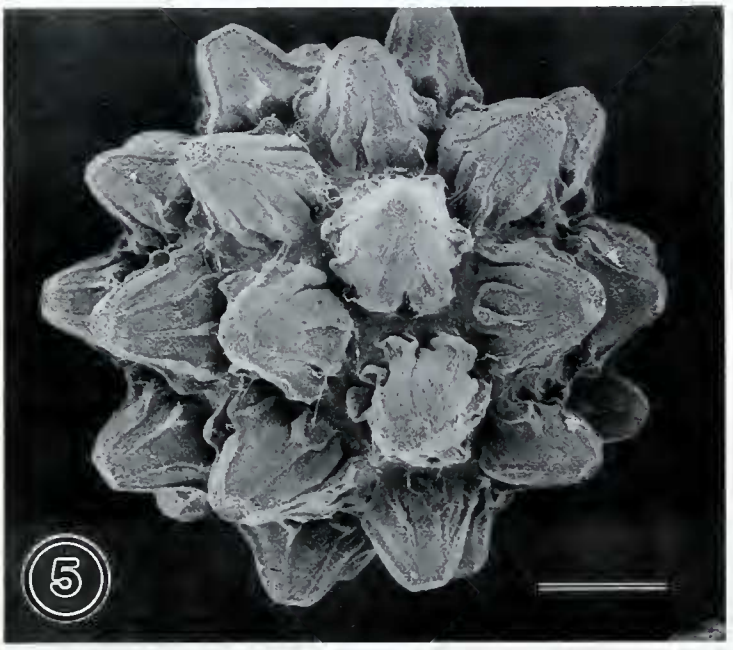
Micropores provide direct access to the vitelline layer

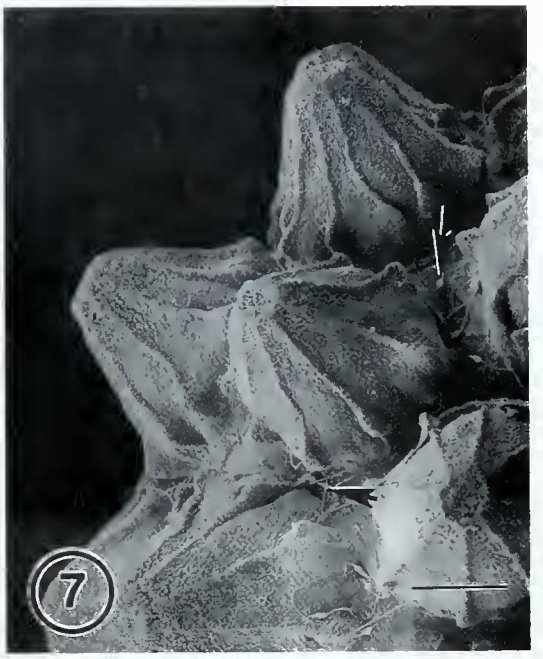
In the two closed cupule species studied (*L. fernaldi* and *L. dentiens*), the area covered by the cupules is unavailable to the sperm, thus focusing them on the intercupule area, where an array of micropores provides direct access to the vitelline layer. Micropores were not found elsewhere on the egg surface. Once again the area available for the fertilizing sperm is only a fraction of the total egg surface.

Spinous hull species have not been studied in detail but preliminary observations in this study suggest that they are equivalent to closed cupule species. The spines of *Chaetopleura apiculata* are hollow and closed to the outside. Observations of hull formation in *Sypharochiton septentriones* (Ashby) (concluded from: Selwood, 1970) also showed the spines to be hollow and closed. Furthermore, Eernisse (1984) has observed closed spines in several spinous hull species, including *Stenoplax fallax* (Carpenter in Pilsbry, 1892).

How do sperm penetrate the egg?

The presence of an acrosome is probably universal among chitons (Buckland-Nicks *et al.*, 1990), although





Figures 5–8: Scanning electron micrographs of fertilized eggs of *Lepidochitona dentiens*.

Figure 5. Fertilized egg of *L. dentiens* demonstrating closed cupules. Scale bar = 40 μm.

Figure 6. Fertilized egg of *L. dentiens* with some cupules removed. No sperm are found inside cupules. Note sperm penetrating hull between cupules (asterisks). Scale bar = 40 μm.

Figure 7. Fertilized egg of *L. dentiens* showing sperm in intercupule area (arrows). Scale bar = 20 μm.

Figure 8. Sperm penetration of *L. dentiens* egg. Sperm anterior filament has entered micropore in intercupule region. Scale bar = 2 μm.

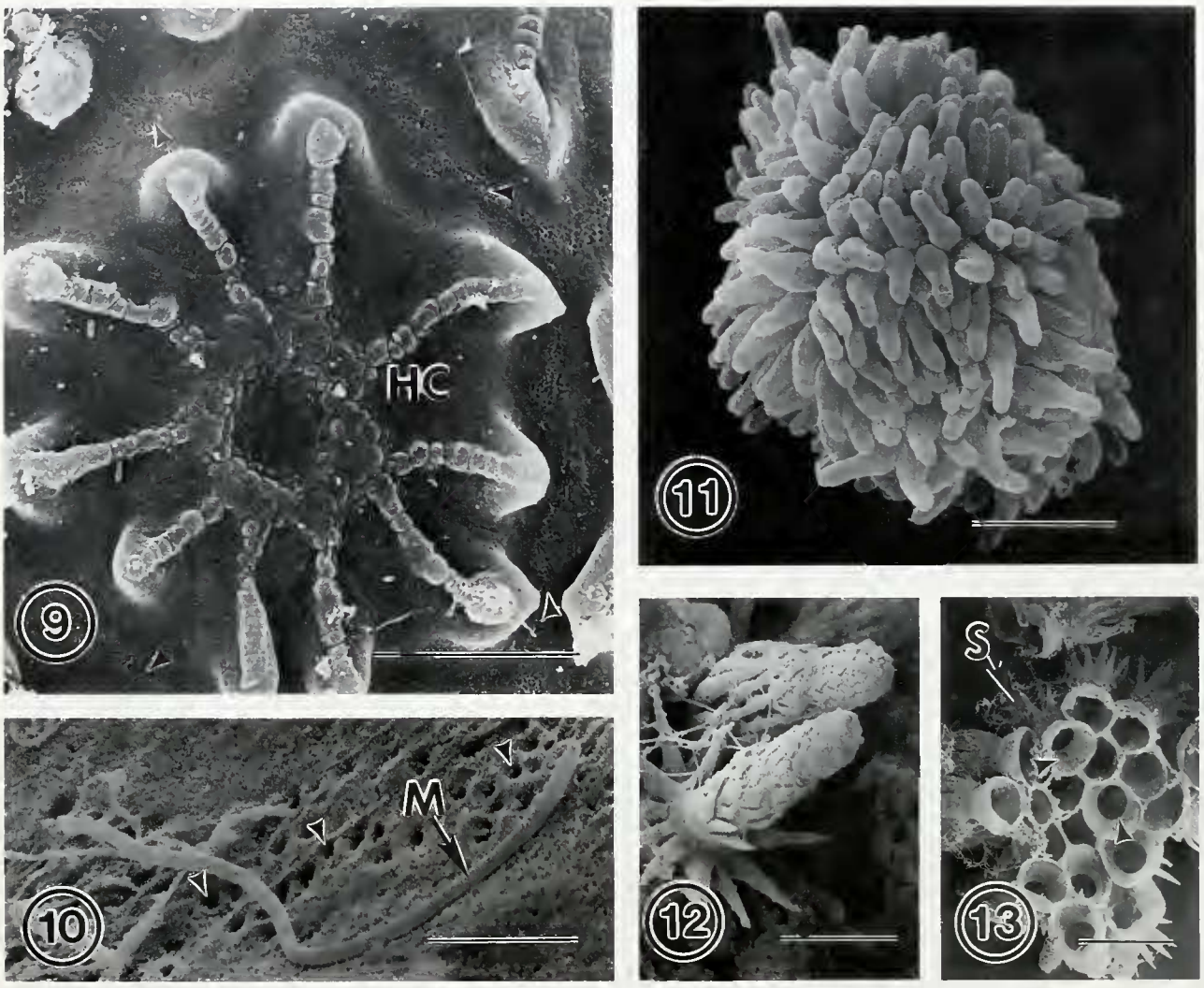


Figure 9. S.E.M. of closed hull cupule (HC) of *Lepidochitona fernaldi* egg, showing reduction typical of brooders. Regular series of micropores is visible in intercupule area (arrowheads). Scale bar = 20 μm.

Figure 10. S.E.M. of *L. fernaldi* egg showing elongate microvillus (arrow), extending from one of a series of micropores in the intercupule region (arrowheads). Scale bar = 2 μm.

Figure 11. S.E.M. of *Chaetopleura apiculata* egg showing spinous hull. Scale bar = 100 μm.

Figure 12. S.E.M. of spine of mature egg of *C. apiculata.* Spines are effectively closed, blocking sperm entry. Scale bar = 20 μm.

Figure 13. S.E.M. of spines of mature egg of *C. apiculata* viewed from the base, showing that they are hollow internally (arrowheads). Note sperm visible only on external surface (S). Scale bar = 40 μm.

the highly reduced form found in the majority of chitons differs markedly from the large, more typical molluscan acrosome found in the primitive Lepidopleurina (Hodgson *et al.*, 1988). Furthermore, there may be key differences in the structure of acrosomes in open and closed cupule species. In the open cupule species *Tonicella lineata,* sperm penetration of hull and vitelline layer apparently involves sequential exhaustion of two Golgi-derived granules in the acrosome (Buckland-Nicks *et al.*, 1988). The apical granule is used up during passage through the hull, whereas the basal granule is used up during passage through the vitelline layer. Any exposed area of the hull, but apparently not the hull cupules themselves, can be penetrated by the sperm; although the majority of sperm are attracted inside the hull cupules. Conversely, in closed cupule species, I have not found sperm penetrating the hull anywhere except in the region of micropores, which enable the sperm to bi-pass the hull and gain direct access to the vitelline layer; although it is not certain yet that sperm are unable to penetrate the hull directly, between micropores. Upon re-examination of the acrosomes of *Chaetopleura apiculata, L. dentiens, L. fer-*

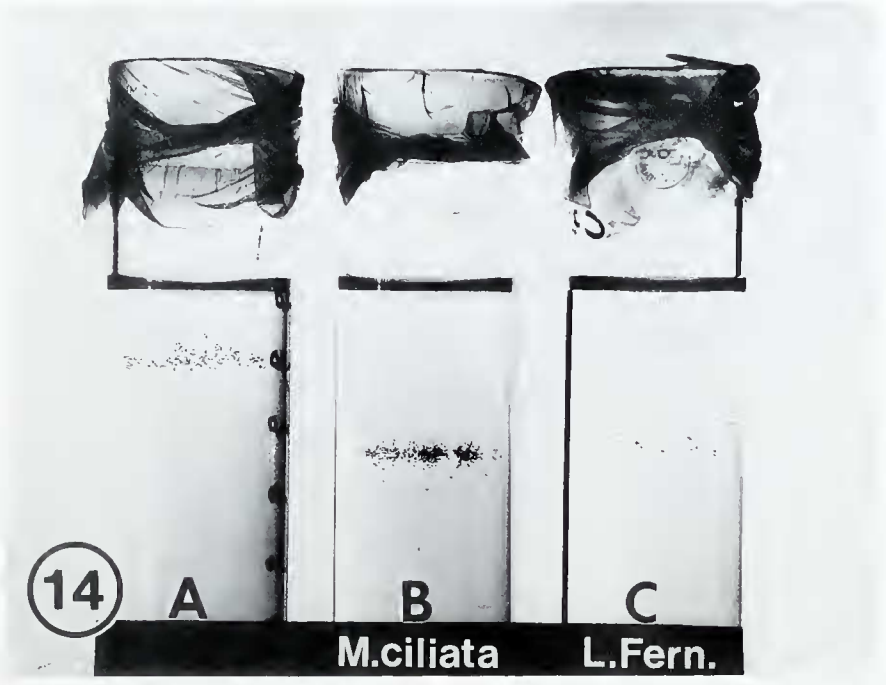


Figure 14. Photograph of eggs of *Lepidochitona fernaldi* and *Mopalia ciliata* centrifuged at 400 G's on Percoll gradients in pyrex tubes. A. *M. ciliata* eggs after 2 min centrifugation. B. *M. ciliata* eggs after 10 min centrifugation. C. *L. fernaldi* eggs after 10 min centrifugation.

naldi, and *L. caverna*, I could only resolve a single granule in the acrosome. This could explain the lack of sperm penetration of the hull except in the region of micropores in *L. fernaldi* and *L. dentiens* (Buckland-Nicks and Eernisse, 1992). Further study will be required to confirm the substructure of acrosomes in open and closed cupule species. Improved fixation methods will have to be devised to clarify these distinctions.

The presence or absence of micropores and the structure of the acrosomes in spinous hull species remain to be discovered.

Specializations of the egg membrane

The direct involvement of microvilli in the fertilization of animals has been documented in vertebrates (Bedford, 1982; Ohta, 1991), as well as many different invertebrates (Longo, 1983; Sato and Osanai, 1983; Fukumoto, 1988), including chitons (Buckland-Nicks *et al.*, 1988b). Richter (1976) and Selwood (1970) point out that elongate microvilli are intimately involved with the secretion of the egg hull in chitons and one would expect to find them within the hull elaborations of all species, during this process. However, the location of elongate microvilli in mature eggs appears to be different in open and closed cupule species, and it may be linked with fertilization. In open cupule species, elongate microvilli are found directly below hull cupules in mature eggs and extend upwards into the vitelline layer and sometimes into the hull (Richter, 1976; Buckland-Nicks *et al.*, 1988b).

In closed cupule species, elongate microvilli are found below the intercupule area. No spinous hull species has been studied in detail with S.E.M., but a photograph taken by D. Eernisse (1984) of the unfertilized egg of *Stenoplax fallax* shows numerous microvilli projecting above the hull in the intercupule area. These and other exposed microvilli likely retract prior to fertilization, and may give rise to the micropores we see in the intercupule area in closed cupule species. Differences in the location of specialized microvilli may indicate variation in the site and mechanism of fertilization in the different chiton groups.

Do hull elaborations act as parachute structures in free-spawners?

Sinking rates of the eggs of *M. ciliata* were much slower than those of *L. fernaldi*. This was not due to differences in overall density, as egg densities were found to be similar. The eggs of *M. ciliata* had a smaller actual volume and a larger effective volume than eggs of *L. fernaldi*. Since density is mass per unit volume, and densities were the same, this indicates that the mass of the *L. fernaldi* egg was slightly greater. I conclude that hull cupules in *M. ciliata* distribute the mass of the egg over a larger effective volume and by doing so they trap a layer of seawater around the egg that reduces effective density and slows sinking rate.

In this manner, the hull cupules are acting as parachute structures (Vogel, 1981).

Some other factors also may influence sinking rates. For example, there may be differential density in eggs, with hull cupules being less dense than the egg core, or perhaps the egg is secreting a low density compound into the cupules, such as a sperm chemoattractant (Miller, 1977). Some ascidians secrete ammonium ions into the cupules, resulting in the production of gas bubbles, which act as a flotation device (Lambert and Lambert, 1978). However, no gas bubbles were observed within the cupules of chiton eggs.

In species where spawned eggs are not dispersed immediately, hull cupules may have other functions such as linking eggs together in chains held by mucus, or sticking eggs on the substrate by their spines (Eernisse, 1988; Eernisse and Reynolds, 1993). In some species, eggs are embedded in a gelatinous mass on the substrate and hull cupules are sometimes reduced, but also they may be retained (Eernisse and Reynolds, 1993). In brooding species, cupules invariably are reduced, sometimes to flattened plates, as in *L. fernaldi* and *L. thomasi* (Eernisse, 1988). Brooding chitons are frequently small, with a restricted space (the pallial grooves) in which to store developing embryos. The reduction in cupule size may correlate with increased potential for brooding, as presumably then more eggs could be packed into such a restricted brood chamber.

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Literature Cited

Al-Hajj, M. A. 1987. Ultrastructural study of spermiogenesis in the chiton *Acanthopleura haddoni* from the Gulf of Aqaba (Red Sea). *Int. J. Invert. Reprod. Dev.* **12:** 295–308.

Bedford, J. M. 1982. Fertilization. Pp. 128–163 in *Reproduction in Mammals, Book 1: Germ Cells and Fertilization,* C. R. Austin and R. V. Short, eds. 2nd ed. Cambridge University Press, Cambridge.

Buckland-Nicks, J., R. Koss, and F.-S. Chia. 1988a. The elusive acrosome of chiton sperm. *Int. J Invert. Reprod Dev.* **13:** 193–198.

Buckland-Nicks, J., R. Koss, and F.-S. Chia. 1988b. Fertilization in a chiton: acrosome-mediated sperm-egg fusion. *Gamete Res.* **21:** 199–212.

Buckland-Nicks, J., F.-S. Chia, and R. Koss. 1990. Spermiogenesis in Polyplacophora, with special reference to acrosome formation (Mollusca). *Zoomorphology* **109:** 179–188.

Buckland-Nicks, J., and D. J. Eernisse. 1993. Ultrastructure of mature sperm and eggs of the brooding hermaphroditic chiton, *Lepidochitona fernaldi* Eernisse 1986, with special reference to the mechanism of fertilization. *J. Exp. Zool.* **265:**567–574.

Eernisse, D. J. 1984. *Lepidochitona* Gray, 1821 (Mollusca: Polyplacophora), from the Pacific Coast of the United States: Systematics and Reproduction. Ph.D. Dissertation. University of California, Santa Cruz. 358 pp.

Eernisse, D. J. 1988. Reproductive patterns in six species of *Lepidochitona* (Mollusca: Polyplacophora) from the Pacific Coast of North America. *Biol. Bull.* **174:** 287–302.

Eernisse, D. J., and P. D. Reynolds. 1993. Polyplacophora. In *Microscopical Anatomy of Invertebrates, vol 5,* F. W. Harrison and A. J. Kohn, eds. Wiley-Liss, New York (In press).

Fukumoto, M. 1988. Fertilization in ascidians: apical processes and gamete fusion in *Ciona intestinalis* spermatozoa. *J. Cell Sci.* **89:** 189–196.

Hodgson, A. N., J. M. Baxter, M. G. Sturrock, and R. T. F. Bernard. 1988. Comparative spermatology of 11 species of Polyplacophora (Mollusca) from the suborders Lepidopleurina, Chitonina and Acanthochitonina. *Proc. R. Soc. London Ser. B* **235:** 161–177.

Lambert, C., and G. Lambert. 1978. Tunicate eggs utilize ammonium ions for flotation. *Science* **200:** 64–65.

Longo, F. J. 1983. Meiotic maturation and fertilization. Pp. 49–89 in *The Mollusca vol. 3, Development,* N. H. Verdonk, J. A. M. van den Biggelaar, and A. S. Tompa, eds. Academic Press, New York.

Miller, R. L. 1977. Chemotactic behavior of the sperm of chitons (Mollusca: Polyplacophora). *J. Exp. Zool.* **202:** 203–212.

Ohta, T. 1991. Initial stages of sperm-egg fusion in the freshwater teleost, *Rhodeus ocellatus ocellatus. Anat. Rec.* **229:** 195–202.

Pearse, J. S. 1979. Polyplacophora. Pp. 27–85 in *Reproduction of Marine Invertebrates, Vol 5, Molluscs: Pelecypods and Lesser Classes,* A. C. Giese and J. S. Pearse, eds. Academic Press, New York.

Pearse, J. S., and R. M. Woollacott. 1979. Chiton sperm: no acrosome? *Am. Zool.* **19:** 956 (abs).

Richter, H-P. 1976. Feinstrukturelle Untersuchungen zur Oogenese der Käferschnecke *Lepidochitona cinereus* (Mollusca, Polyplacophora). *Helgol. Wiss. Meeresunters.* **28:** 250–303.

Russell-Pinto, F., C. Azevedo, and T. Barandela. 1983. Fine structure of the spermatozoa of *Chiton marginatus* (Mollusca: Amphineura), with special reference to nucleus maturation. *Gamete Res.* **8:** 345–355.

Russell-Pinto, F., C. Azevedo, and E. Oliveira. 1984. Comparative ultrastructural studies of spermiogenesis and spermatozoa in some species of Polyplacophora (Mollusca). *Int. J. Invert. Reprod. Dev.* **7:** 267–277.

Sakker, E. R. 1984. Sperm morphology, spermatogenesis and spermiogenesis of three species of chitons (Mollusca, Polyplacophora). *Zoomorphology* **104:** 111–121.

Sato, M., and K. Osanai. 1983. Sperm attachment and acrosome reaction on the surface of the polychaete, *Tylorrhynchus heterochaetus. Biol. Bull.* **178:** 101–110.

Selwood (née Bedford), L. 1970. The role of the follicle cells during oogenesis in the chiton *Sypharochiton septentriones* (Ashby) (Polyplacophora, Mollusca). *Z. Zellforsch.* **104:** 178–192.

Vogel, S. 1989. *Life in Moving Fluids: The Physical Biology of Flow,* 3rd ed. Princeton University Press, New Jersey. 352 pp.